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Can concurrent abnormalities in free light chains and immunoglobulin concentrations identify a target population for immunoglobulin trials in sepsis?

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Abstract

Objective: Light chains kappa (κ) and lambda (λ) are immunoglobulin constituents but also circulate independently in blood as free light chains (FLC). We investigated whether a concomitant abnormality in FLC and immunoglobulin levels could identify a high risk of death sepsis subpopulation to inform future IVIg trials. We tested whether light chain allelic inclusion occurs in circulating B cells.

Design: Prospective cohort study

Setting: Adult general intensive care units (ICU)

Patients: Adult sepsis patients without any documented immune comorbidity.

Interventions: None

Measurements and main results: Serum total FLC, IgG, IgA and IgM were measured on ICU Days 1, 3 and 7. Population normal ranges defined normal and abnormal categories. Logistic regression models tested any independent relationship between high-FLC, immunoglobulins and hospital mortality. CD19 B cell subsets expressing cell surface κ and λ were quantified by flow cytometry; their frequencies were compared against healthy subjects and correlation assessed against FLC concentrations. On ICU Day 1, high-FLC λ and high-FLC κ were seen in 46.5% and 75.3% of the study cohort (n=101). Low immunoglobulin levels were commonplace (45.5%) on ICU admission. ICU admission day FLC and immunoglobulin concentrations were significantly correlated. Septic patients had significantly more CD19 B cells expressing both κ and λ compared with healthy controls (median (IQR) 4.1% (2.4, 11.0) vs. 1.3% (1.2, 2.9) respectively; p=0.0001); these correlated with FLC concentrations.

Conclusions: To our knowledge, abnormalities and associations of FLC in critically ill adults with sepsis have not been previously reported. The additional prognostic value of FLC λ and the significance of allelic inclusion in B cells in sepsis require further investigation.

Introduction

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection (1). Attempts to favorably modulate a dysregulated immune response with intravenous immunoglobulin therapy (IVIg) failed to improve outcomes, as these trials potentially did not target treatment responsive subpopulations (2-4). Studies that help to enrich sepsis subpopulations based on abnormalities in immunoglobulin biology could inform future IVIg trial design, as new trials are being planned (5). We recently tested whether low immunoglobulin G (IgG) levels on the day of sepsis diagnosis identified a higher risk of death subpopulation (6). Despite a high prevalence of low IgG levels, this abnormality alone did not identify mortality risk.

In health, circulating immunoglobulin consists of heavy chains bound by disulfide bonds to either kappa (κ) or lambda (λ) light chains. These light chains are also measurable as circulating free light chains (FLC), separate from immunoglobulin molecules. Raised FLC levels are associated with sepsis risk factors such as low-grade inflammation, increasing age and renal dysfunction (7-9). Altered FLC concentrations have been reported in a pilot study performed on 21 ICU patients with sepsis (10).

Since reduced immunoglobulin and raised FLC concentrations are both associated with sepsis, we investigated whether their concomitant abnormality could enrich a sepsis population for IVIg trials (Table 1) (11, 12). Ideally, enrichment markers should forecast the likely patient outcome irrespective of baseline risk, and should also have biological plausibility (6, 11). Several observations highlight such plausibility for concomitant FLC and immunoglobulin abnormalities in sepsis, in addition to the known associations of FLCs described above (7-10). In health, excess light chains are present in the perinuclear space to prevent intracellular heavy chain aggregation prior to immunoglobulin assembly, and are secreted as FLC after immunoglobulin assembly (13). Thus, impaired immunoglobulin assembly in sepsis could result in raised FLC and low immunoglobulin levels (2, 3, 14). We also considered whether an altered stoichiometry of light chain production could contribute to raised FLC concentrations. Rules of allelic exclusion permit only a single immunoglobulin

specificity by each B cell. However, we recently reported that acute inflammation in autoimmune diseases can result in simultaneous expression of both κ and λ light chains by a single B cell. Thus, light chain allelic inclusion in the context of acute inflammation may result in production of excess FLC (15). Against this background, after describing longitudinal changes in FLC and immunoglobulin levels in sepsis, we tested whether concomitant abnormalities in FLC and immunoglobulin levels on ICU admission day are independently associated with hospital mortality. We also examined surface κ and λ LC expression on B cells.

Materials and Methods

Study design and setting

This prospective observational cohort study was performed in the general medical-surgical ICUs of an university hospital. Ethics committee approval was obtained prior to start of recruitment (10/H0807/81 and 12/LO/0326; Camberwell St Giles Committee, London, UK). Clinical management was at the discretion of the attending physician.

Study population

Consecutive patients with sepsis within the first 12 hours following ICU admission between 05/2011 and 01/2015 were included. Sepsis was identified using the old definition as evidence of two or more systemic inflammatory response syndrome (SIRS) criteria, with proven or suspected infection and at least one organ system dysfunction (cardiovascular, respiratory, renal, hematologic or metabolic) (16). Exclusion criteria included patients <18 years, those with congenital hypogammaglobulinemia, known protein-losing enteropathies, nephrotic syndrome, neoplastic or proliferative hematologic diseases; those having received IVIg therapy in the last 3 months; those receiving high-dose corticosteroid therapy; ongoing blood loss (defined by blood transfusion requirement > 2 units/24 hour period); retroviral disease; and immune dysfunction as defined by Acute Physiology and Chronic Health Evaluation (APACHE) II score co-morbidities (17). We have recently published other details on B cell changes in sepsis from this population, using the same cohort (14).

Clinical data, blood sampling and flow cytometry

Baseline demographic data, infection details, and variables to record SIRS, APACHE II (17) and Sepsis-related Organ Failure Assessment (SOFA) scores (18) were collected. Blood samples were collected within 12 hours of ICU entry on admission day (D1), on day 3 (D3), and on day 7 (or on the day of demise or discharge if these events occurred earlier). After clot separation and centrifugation (3000 rpm; 10 minutes; 4°C), serum samples were stored at -70°C. Blinded batch analysis was performed for immunoglobulin and FLC levels to reduce inter-assay variation. Immunoglobulin molecules (total IgG, IgA and IgM) (Dade Behring Nephelometer, Marburg, Germany) and serum FLC using polyclonal sheep antisera (Freelite™, Binding Site, Birmingham, UK) were analysed on a SPA plus analyser (Binding Site). The free light chain ratio (FLCR) was derived as the ratio of FLC κ to FLC λ .

Flow cytometry was used to ascertain light chain allelic inclusion in circulating B cells using a Canto-A flow cytometer (Becton Dickinson (BD), Franklin Lakes, New Jersey). PBMCs were isolated within 4 hours of blood sampling using Ficoll density centrifugation. Anti-human antibodies, clone and company are reported in parenthesis. Anti-CD19 (PerCP Cy5.5; HB19; BD); anti- κ LC (ACP-H7; G20-193; BD); anti- λ LC (Pacific Blue; MHL-38; BioLegend) and Live/Dead Fixable Aqua (Life Technologies) antibodies were used for cell surface expression of CD19, LC- κ and LC- λ , respectively. Gating during analysis of flow cytometry standard (FCS) files were achieved using isotype controls, fluorescence minus-one controls, and/or single stain controls.

Derivation of abnormal categories

The reference normal ranges for an adult general population in the UK are IgG (6.1 – 16.2) g/l, IgA (0.8 – 5.0) g/l, IgM (0.4 – 2.4) g/l, FLC λ (5.7 – 26.3) mg/l, FLC κ (3.3 – 19.4) mg/l and FLCR (0.26 – 1.65). The upper and lower limits of these normal ranges were used to define normal and abnormal categories. These categorizations allowed generation of prevalence data, and comparison of the proportions of survivor and non-survivor sepsis patients with each abnormality.

Sample size estimation

Sample size was estimated using precision estimates for IgG distribution (19), as IgG is the most often altered immunoglobulin in sepsis (6). One hundred patients were required to achieve a margin of error of 0.74 g/L of IgG measurement based on the population standard deviation of 3.72 g/L observed in the first 30 patients enrolled.

Statistics

Continuous data are presented as mean and standard deviation (SD) when normally distributed, and as median and interquartile range (IQR) when not. Frequency and percentages are presented for categorical data.

Two sets of comparisons described longitudinal changes in FLC. First, we compared proportions of high FLCs and concentrations of FLCs between survivors and non-survivors on days 1, 3 and 7. Second, we compared how proportions of high FLCs and temporal changes in concentration varied between survivors and non-survivors using paired tests. A similar approach was used to describe changes in immunoglobulins.

To report univariate associations and to inform logistic regression models, a pairwise correlation matrix was generated for admission day FLC, FLCR, IgG, IgA, IgM, white cell count, C-reactive protein (CRP), total SOFA and APACHE II score. Bonferroni corrections were performed for multiple comparisons. Two analyses were performed to assess any association between a concomitant admission day immunoglobulin and FLC abnormality and mortality. Stratified analyses were performed as shown in Table 1. Two logistic regression models were then used with acute hospital mortality as the outcome: model 1 had high-FLC λ , total APACHE II score, hypo-IgG, and hypo-IgM as binary covariates, while model 2 had high-FLC κ , total APACHE II score, hypo-IgG, and hypo-IgM as binary covariates.

B cell subsets are reported as a proportion of the live CD19 positive B cell population. Proportions were compared between healthy controls and sepsis. All analyses were performed using GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA,

www.graphpad.com) and/or Stata/SE Version 13.0 (StataCorp LP, College Station, TX).

Reported p values are two sided with p values <0.05 representing statistical significance.

Results

Study cohort

Cohort characteristics by hospital survival status are summarized in Table 2. Acute hospital mortality was 26.7%. Non-survivors were older and had greater illness severity as evidenced by greater APACHE II and SOFA scores. The respiratory tract was the commonest infection site in both survivors and non-survivors. In two patients, the site of infection could not be confirmed.

Longitudinal changes in FLC κ and FLC λ

The prevalence of high FLC κ was similar in survivors and non-survivors on D1 ($p=0.45$); D3 ($p=0.18$) and DF ($p=0.75$). No significant differences were seen in corresponding FLC κ concentrations at any timepoint (Figure 1a). The proportion of sepsis patients with abnormal FLC λ was significantly higher in non-survivors only on D1 (74.1% vs. 36.5%; $p=0.001$) but not D3 ($p=0.24$) or DF ($p=0.11$). There was an increase in corresponding FLC λ concentrations over time in both survivors and non-survivors ($p<0.05$). However, when comparing FLC λ concentrations on days 1, 3 and DF between survivors and non-survivors, there were no differences at any time point (Figure 1b).

Longitudinal changes in FLCR

Despite recruiting septic patients without any documented immune comorbidity, 29.7% had a high FLCR. Prevalence was higher in survivors on D1 (36.5% vs 11.1%, $p=0.01$). This ratio decreased on D3 compared to D1 in both survivors and non-survivors ($p<0.05$; Figure 1c). Analyses restricted to patients with a normal FLCR showed that the proportion of patients with a high FLC λ was more common in non-survivors on D1 (75.0% vs. 40.4%; $p=0.007$) whereas the proportion with high FLC κ was similar between survivors and non-survivors (63.8% vs. 83.3%; $p=0.11$).

Longitudinal changes in immunoglobulin subtypes

Although hypo-IgG was the most prevalent immunoglobulin abnormality, there was no difference in hypo-IgG by hospital survival status at D1 ($p=0.22$); D3 ($p=0.92$) and DF ($p=0.07$). The nadir for IgG was seen on D3. The fall in D3 in eventual non-survivors was 8 times greater than that seen in survivors (median (IQR) in survivors was -0.121 (-1.28 ; 0.87) and in non-survivors was -0.80 (-1.28 ; -0.3); $p=0.02$). Compared to D1, D7 IgG concentrations rose only in survivors ($p=0.004$; Figure 1d). Similar to IgG concentrations, there were no difference in hypo-IgM by hospital survival status at any time point. The nadir for IgM was on D3, ($p=0.02$ in non-survivors; Figure 1e). Hypo-IgA was the least prevalent (6.9%) and observed in only 7 survivors (Figure 1f).

Associations of FLC and immunoglobulin

FLC κ was significantly correlated with FLC λ , FLCR, IgG and serum creatinine. FLC λ was significantly correlated with FLC κ , IgG, IgA and serum creatinine. IgG and IgM were significantly correlated. FLCs were not correlated to markers of illness severity (APACHE II and SOFA) nor to acute inflammation (CRP and WBC) (eFigure 1).

High FLC λ is associated with mortality

Using the concept presented in Table 1, the stratified analyses highlighted higher mortality only in patients with high FLC λ , irrespective of their hypo-IgG or hypo-IgM status (Figure 2a and Table-3). Hypo-IgA was not included in our logistic regression models assessing high FLC mortality relationship as it occurred only in survivors on Day 1. APACHE II score accounted for illness severity and differences in age between survivors and non-survivors reported in Table 2. In *model 1*, hospital mortality was significantly associated with the APACHE II score and high FLC λ . The increase in OR (95% CI) for hospital mortality per unit increase in APACHE II score was 1.2 (1.1 – 1.4; $p<0.001$) and 4.0 (1.3–12.4) for high FLC λ ($p=0.017$). In *model 2*, hospital mortality was significantly associated only with the APACHE II score; the OR (95% CI) per unit increase in APACHE II score was 1.2 (1.1–1.4; $p<0.001$) (eFigure2).

Allelic inclusion in B cells and associations

The gating strategy for identification of light chain allelic inclusion is shown in eFigure3. The characteristics of the 21 sepsis patient samples used for flow cytometry are summarized in eTable1. CD19 B cells expressing cell surface LC κ or LC λ , expressed as a proportion of alive CD19 population and as a ratio of CD19 κ to CD19 λ were similar in sepsis patients and healthy controls. Septic patients had a significantly higher proportion of CD19 B cells expressing both LC κ and LC λ (CD19^{KL}) than B cells taken from healthy subjects (median (IQR) 4.1% (2.4, 11.0) vs. 1.3% (1.2, 2.9) respectively; $p=0.0001$; Figure2b). A significant correlation existed between the frequencies of these CD19^{KL} cells and the corresponding FLC concentrations (Figure 2c). A significant correlation was only observed between FLC λ and CD19 B cells expressing cell surface LC λ (Figure 2c).

Discussion

High FLC κ , high FLC λ and high FLCR are common in septic patients admitted to critical care without known immune comorbidities. The presence of a high FLC λ increases the risk of death, even after adjusting for APACHE II score, despite high FLC κ being the more common abnormality. The combination of high FLC and low immunoglobulins on ICU admission day was more prevalent in a greater proportion of non-survivors. Consistent with the published literature (6), we found that hypo-IgG and hypo-IgM were common in sepsis on D1, reaching a nadir on D3, and that more survivors incremented immunoglobulin levels over time. These findings increase the external validity of our results.

The approach presented in Table-1 is discussed in the context of our results to inform trial design. IVIg treatment potentially restores normal serum immunoglobulin concentrations in patients with low IgG levels, in addition to contributing to immunomodulation (3). In clinical trials, the IVIg treatment effect will vary by the baseline risk of death, i.e. a heterogeneity in treatment effect (20, 21). Thus, enriching sepsis patients with a higher risk of death concomitant with a greater likelihood of treatment response inferred by low immunoglobulins on admission may be more likely to result in a positive outcome if the strategy is indeed effective (11, 12). In this study, we position FLC λ as a potential biomarker within the causal

pathway from immunoglobulin alterations to death in sepsis patients, after accounting for illness severity. High FLC λ identifies a higher risk of death sepsis population. The prevalence of high FLC λ within our sepsis cohort was 46.5%, with 74% of non-survivors having this abnormality. These concepts could be easily validated using data from ongoing trials (5).

We observed major abnormalities in immunoglobulin levels without an increase in the risk of death; this finding is consistent with our previous systematic review (6). Dissecting the relative contributions of altered immunoglobulin production, impaired endothelial recycling, and immunoglobulin consumption for opsonization during sepsis is challenging due to the lack of a well characterized regulatory loop for immunoglobulin homeostasis (22). There are no mechanistic studies to date explaining immunoglobulin kinetics in sepsis. Our first novel finding is that FLCs are raised with a high FLC λ being associated with an increased risk of hospital mortality after adjustment for illness severity. The kidneys maintain circulating FLC homeostasis via glomerular clearance, proximal tubule endocytosis and catabolism (23). Impairments in renal function seen in sepsis could increase FLC concentrations, and this is supported by the positive correlation found between serum FLC and creatinine levels. The increase in FLC could also be due in part to impaired immunoglobulin assembly. A pilot study of 21 patients with pneumonia and septic shock found that FLC concentrations did not differ by IgG level (10). However, this study neither reported longitudinal changes in FLC by survival status, nor mechanisms. The FLCR was similar to our cohort, providing further external validity to our results (10).

Important unanswered questions are the mechanisms by which FLC concentrations increase in sepsis, and how this affects sepsis biology in terms of illness severity or outcome. The increased proportion of CD19^{K λ} dual cells in sepsis and their association with raised FLC concentrations is another novel finding that warrants further investigation. In healthy adults, CD19^{K λ} dual cells represent only 0.5% of circulating B cells (24); the rise seen in our sepsis cohort could be a random simultaneous rearrangement of both light chain loci, but is most likely to represent allelic inclusion due to impaired kappa deleting element (KDE) activity or a transient phase during light chain revision (24).

There are several limitations to our study. We have not investigated mechanisms behind these novel findings. We cannot ascertain whether the FLC abnormalities were present before the onset of sepsis nor whether FLCs represent a risk factor for sepsis, or mortality irrespective of sepsis (8). We accounted for these challenges by studying patients without immune comorbidity and by showing the independent effect of high FLC λ on hospital mortality after adjusting for APACHE II score. These novel observations should be replicated in other sepsis cohorts.

As FLC concentrations are linked to immunoglobulin assembly in the literature, our findings imply that this FLC-immunoglobulin relationship makes FLC a potential biomarker for simultaneous prognostic and predictive enrichment of sepsis patients for IVIg therapy trials (11). This hypothesis could be confirmed using samples from existing trial data (5). Further questions raised include the contribution of elevated FLC to renal dysfunction in sepsis, as observed in myeloma patients (23). Raised FLC levels could also contribute to the prolonged neutrophil life span in sepsis (25), acting through enhanced tyrosine phosphorylation (26). The mechanisms involved in B-cell allelic inclusion in sepsis needs exploring.

Conclusions

In a well-characterized cohort of critically ill adults with sepsis without any immune comorbidity, we report a high prevalence of FLC abnormalities associated with immunoglobulin subtype abnormalities and offering prognostic utility. We show evidence of light chain allelic inclusion in B cells. The roles in sepsis pathology and as a putative biomarker for IVIg therapy warrants further study.

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References

1. Singer M, Deutschman CS, Seymour CW, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA* 2016;315(8):801-810.
2. Hotchkiss RS, Monneret G, Payen D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. *Nat Rev Immunol* 2013;13(12):862-874.
3. Shankar-Hari M, Spencer J, Sewell WA, et al. Bench-to-bedside review: Immunoglobulin therapy for sepsis - biological plausibility from a critical care perspective. *Crit Care* 2012;16(2):206.
4. Marshall JC. Why have clinical trials in sepsis failed? *Trends Mol Med* 2014;20(4):195-203.
5. Welte T, Dellinger RP, Ebel H, et al. Concept for a study design in patients with severe community-acquired pneumonia: A randomised controlled trial with a novel IGM-enriched immunoglobulin preparation - The CIGMA study. *Respir Med* 2015;109(6):758-767.
6. Shankar-Hari M, Culshaw N, Post B, et al. Endogenous IgG hypogammaglobulinaemia in critically ill adults with sepsis: systematic review and meta-analysis. *Intensive Care Med* 2015;41(8):1393-1401.
7. Dispenzieri A, Katzmann JA, Kyle RA, et al. Use of nonclonal serum immunoglobulin free light chains to predict overall survival in the general population. *Mayo Clin Proc* 2012;87(6):517-523.
8. Anandram S, Assi LK, Lovatt T, et al. Elevated, combined serum free light chain levels and increased mortality: a 5-year follow-up, UK study. *J Clin Pathol* 2012;65(11):1036-1042.
9. Bradwell AR. Serum free light chain measurements move to center stage. *Clin Chem* 2005;51(5):805-807.
10. Taccone FS, Stordeur P, De Backer D, et al. Gamma-globulin levels in patients with community-acquired septic shock. *Shock* 2009;32(4):379-385.
11. Shankar-Hari M, Rubinfeld GD. The use of enrichment to reduce statistically indeterminate or negative trials in critical care. *Anaesthesia* 2017;72(5):560-565.
12. Prescott HC, Calfee CS, Thompson BT, et al. Toward Smarter Lumping and Smarter Splitting: Rethinking Strategies for Sepsis and Acute Respiratory Distress Syndrome Clinical Trial Design. *Am J Respir Crit Care Med* 2016;194(2):147-155.
13. Nakano T, Matsui M, Inoue I, et al. Free immunoglobulin light chain: its biology and implications in diseases. *Clin Chim Acta* 2011;412(11-12):843-849.
14. Shankar-Hari M, Fear D, Lavender P, et al. Activation-Associated Accelerated Apoptosis of Memory B Cells in Critically Ill Patients With Sepsis. *Crit Care Med* 2017;45(5):875-882.
15. Fraser LD, Zhao Y, Lutalo PM, et al. Immunoglobulin light chain allelic inclusion in systemic lupus erythematosus. *Eur J Immunol* 2015;45(8):2409-2419.
16. Levy MM, Fink MP, Marshall JC, et al. 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Crit Care Med* 2003;31(4):1250-1256.

17. Knaus WA, Draper EA, Wagner DP, et al. APACHE II: a severity of disease classification system. *Crit Care Med* 1985;13(10):818-829.
18. Vincent JL, Moreno R, Takala J, et al. The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine. *Intensive Care Med* 1996;22(7):707-710.
19. Arnold DM, Burns KE, Adhikari NK, et al. The design and interpretation of pilot trials in clinical research in critical care. *Crit Care Med* 2009;37(1 Suppl):S69-74.
20. Iwashyna TJ, Burke JF, Sussman JB, et al. Implications of Heterogeneity of Treatment Effect for Reporting and Analysis of Randomized Trials in Critical Care. *Am J Respir Crit Care Med* 2015;192(9):1045-1051.
21. Welton NJ, Soares MO, Palmer S, et al. Accounting for Heterogeneity in Relative Treatment Effects for Use in Cost-Effectiveness Models and Value-of-Information Analyses. *Med Decis Making* 2015;35(5):608-621.
22. Junghans RP. IgG Biosynthesis: No "Immunoregulatory Feedback". *Blood* 1997;90(10):3815-3818.
23. Basnayake K, Stringer SJ, Hutchison CA, et al. The biology of immunoglobulin free light chains and kidney injury. *Kidney Int* 2011;79(12):1289-1301.
24. Giachino C, Padovan E, Lanzavecchia A. kappa+lambda+ dual receptor B cells are present in the human peripheral repertoire. *J Exp Med* 1995;181(3):1245-1250.
25. Taneja R, Parodo J, Jia SH, et al. Delayed neutrophil apoptosis in sepsis is associated with maintenance of mitochondrial transmembrane potential and reduced caspase-9 activity. *Crit Care Med* 2004;32(7):1460-1469.
26. Cohen G, Rudnicki M, Deicher R, et al. Immunoglobulin light chains modulate polymorphonuclear leucocyte apoptosis. *Eur J Clin Invest* 2003;33(8):669-676.
27. Lee WL, Slutsky AS. Sepsis and endothelial permeability. *N Engl J Med* 2010;363(7):689-691.

Figure and Table Legends

Table legends

Table 1: Potential relationships between immunoglobulins and free light chains abnormalities in sepsis patients

We have considered the observable scenarios depicting acute changes in FLC and immunoglobulin concentrations, their possible underlying biological explanations and FLC as a surrogate marker linked to immunoglobulin biology with potential to enrich or stratify sepsis patients (11) in intravenous immunoglobulin trials. [The rationale for this approach and the hypothetical scenarios presented here is that FLC levels are considered a valid surrogate marker of immunoglobulin production and potentially related to outcome, which places FLC as a biomarker in the causal chain between sepsis, immunoglobulins and death. We tested four possible combinations of two variables FLC and immunoglobulins, categorised into normal and abnormal based on their normal clinical laboratory range. Low immunoglobulins in sepsis could represent excess immunoglobulin consumption or extravasation of immunoglobulins\(27\) or impaired IgG recycling\(3\), or a normal immunoglobulin consumption but with impaired immunoglobulin assembly due to B cell abnormalities in sepsis\(14\). High FLC in sepsis could be related to either excess production related to sepsis related inflammation or due to allelic inclusion of light chains in B cells\(13, 15\). Enrichment principles are further explored in these reviews \(11, 12\). Abbreviations: FLC – Free light chains; IVIg – intravenous immunoglobulins](#)

Table 2: Cohort characteristics

Table shows the salient sepsis specific and generic case mix of study cohort. ¹continuous data presented as mean and standard deviation when normally distributed; ²Categorical data-presented as number (proportion); ³continuous data presented as median and inter quartile range when not normally distributed. APACHE – Acute physiology and chronic health evaluation; WCC – white cell count; CRP – C-reactive protein; SOFA- Sequential organ failure assessment; LOS – Length of stay; FLC = free light chains; FLCR – Free light chain kappa/lambda ratio. IgG – immunoglobulin G; IgM – immunoglobulin M; IgA – immunoglobulin A. [The admission day concentrations of immunoglobulins, FLC and FLCR are summarized](#)

Table-3: Prevalence of different scenarios by survival status in the overall cohort

Normal/raised FLC and normal/low immunoglobulin categories were derived as described under *Derivation of abnormal categories* section. Then for each of the scenarios described in table-1 the proportion of patients meeting each scenario within survivors and non-survivors were estimated. These analyses were performed as the prevalence of each scenario and their potential association to survival status will inform future trial design. As our study was powered using precision sample size estimation of Immunoglobulin G concentrations, no formal statistical tests were done for comparisons between groups. There were 27 non-survivors in this cohort. Abbreviations: IgG – immunoglobulin G; IgM – immunoglobulin M; FLC κ = free light chain kappa; FLC λ = free light chain lambda

Figure legends

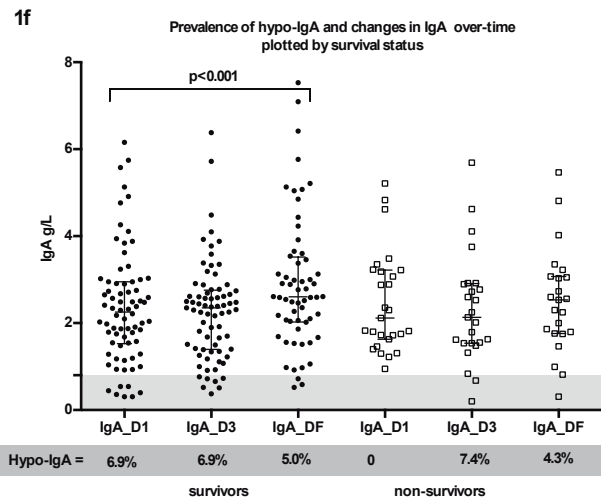
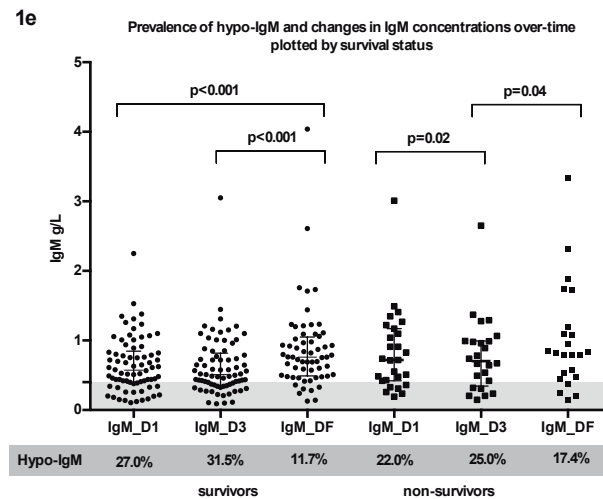
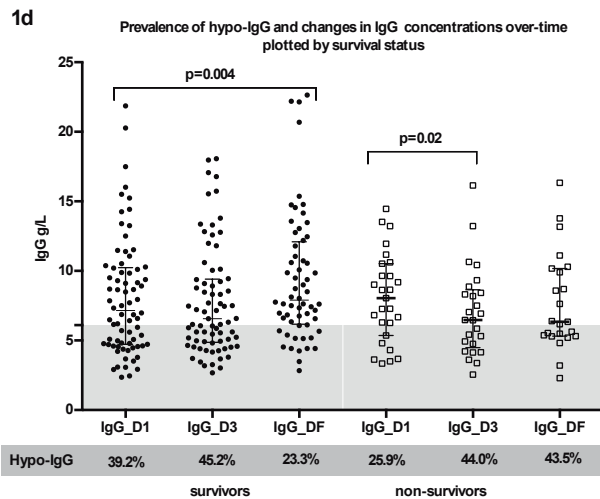
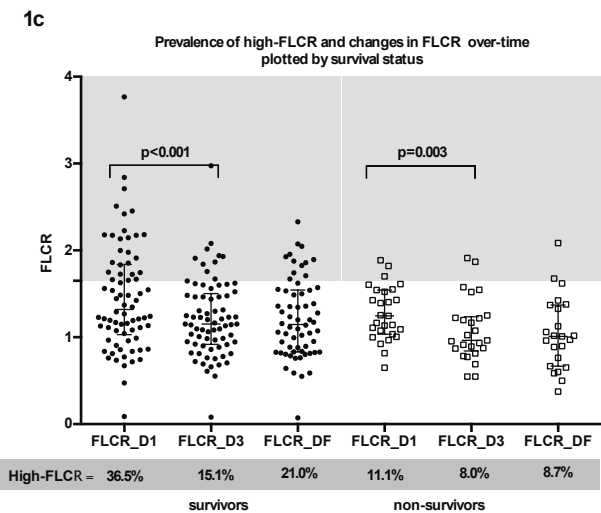
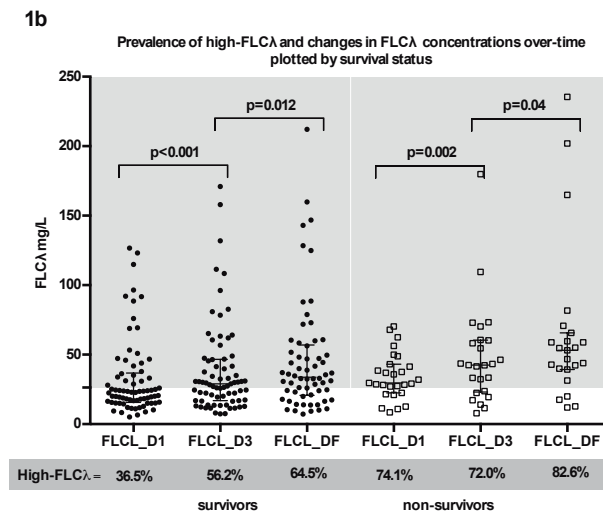
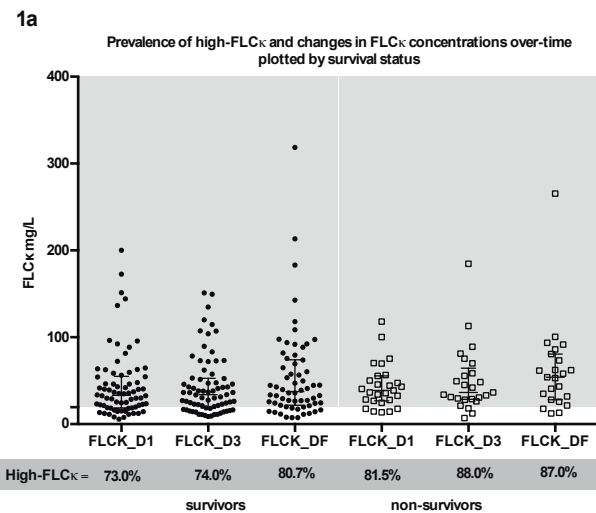
Figure 1: Changes in FLC and Immunoglobulins over time by survival status

Scatter plots showing all data points and median (IQR). D1= ICU admission day, D3= third day following admission and DF=Final sampling day. The shaded regions in 1a, 1b and 1c represents high-FLC category and in 1d, 1e and 1f represent hypo-Ig category. Comparisons by survival status were done using t-tests and within survivors and non-survivors were done using paired t-tests or non-parametric equivalent. **[1a]** FLC κ showed no significant differences in high-FLC κ prevalence and in FLC κ concentrations over time. **[1b]** FLC λ showed significant differences in high-FLC λ prevalence between survivors and non-survivors on D1 ($p=0.001$). The FLC λ concentrations increased significantly over time in survivors and non-survivors. **[1c]** FLCR showed significant differences in high-FLCR prevalence between survivors and non-survivors on D1 ($p=0.01$); D3 ($p=0.51$) and DF ($p=0.34$) and that there was a significant drop in FLCR between D1 and D3 in both survivors and non-survivors. **[1d]** IgG showed no significant differences in hypo-IgG prevalence between survivors and non-survivors on D1 ($p=0.22$); D3 ($p=0.92$) and DF ($p=0.07$). In survivors D1 vs. D3 was not significant ($p=0.57$). In non-survivors D1 vs. DF ($p=0.82$) and D3 vs. DF ($p=0.44$) were not significant. **[1e]** IgM showed no significant differences in hypo-IgM prevalence between survivors and non-survivors on D1 ($p=0.63$); D3 ($p=0.62$) and DF ($p=0.49$). The survivors had a higher IgM by the DF when compared to D1 and D3 levels. The D3 IgM concentrations were significantly

lower than D1 only in non-survivors ($p=0.02$) vs. $p=0.43$ in survivors. **[1f]** On D1, seven survivors had hypo-IgA. The IgA concentrations were similar in survivors and non-survivors on D1, D3 and DF. In survivors D1 vs. D3 was not significant $p=0.50$. In non-survivors D1 vs. D3 ($p=0.28$), D1 vs. DF ($p=0.82$) and D3 vs. DF ($p=0.57$) were not significant.

Figure 2: Stratified analyses of mortality by high-FLC and immunoglobulin status and Allelic inclusion in B cells in sepsis

[2a] The prevalence of FLC and immunoglobulin abnormality and their association to hospital mortality are reported. These analyses show outcomes in the scenarios A = Normal FLC + Normal immunoglobulin; B = Normal FLC + Low immunoglobulins; C = High-FLC + Normal immunoglobulin and D = High-FLC + Low immunoglobulin; **[2b]** Scatter plot showing all data points, the mean and 95% confidence intervals. Mann-Whitney U tests were used to generate p values by comparing sepsis ($n=21$) and healthy samples ($n=15$). The proportions of CD19^{κλ} expressing B cells were seen significantly more common in sepsis patients. SS – Sepsis; H – Healthy; κ– Igκ on CD19 cells; λ– Igλ on CD19 cells; CD19^{κλ} – CD19 expressing both Igκ and Igλ on cell surface. **[2c]** Statistically significant positive correlation was seen between the proportion of CD19^{κλ} dual B cells in sepsis patients and the corresponding admission day FLCλ and FLCκ concentrations. [Statistically significant positive correlation was also seen between the proportion of CD19^λ B cells in sepsis patients and the corresponding admission day FLCλ concentrations but not with CD19^κ B cells and the corresponding FLCκ concentrations. We used linear regression to derive \$R^2\$ when there was significant correlation.](#)



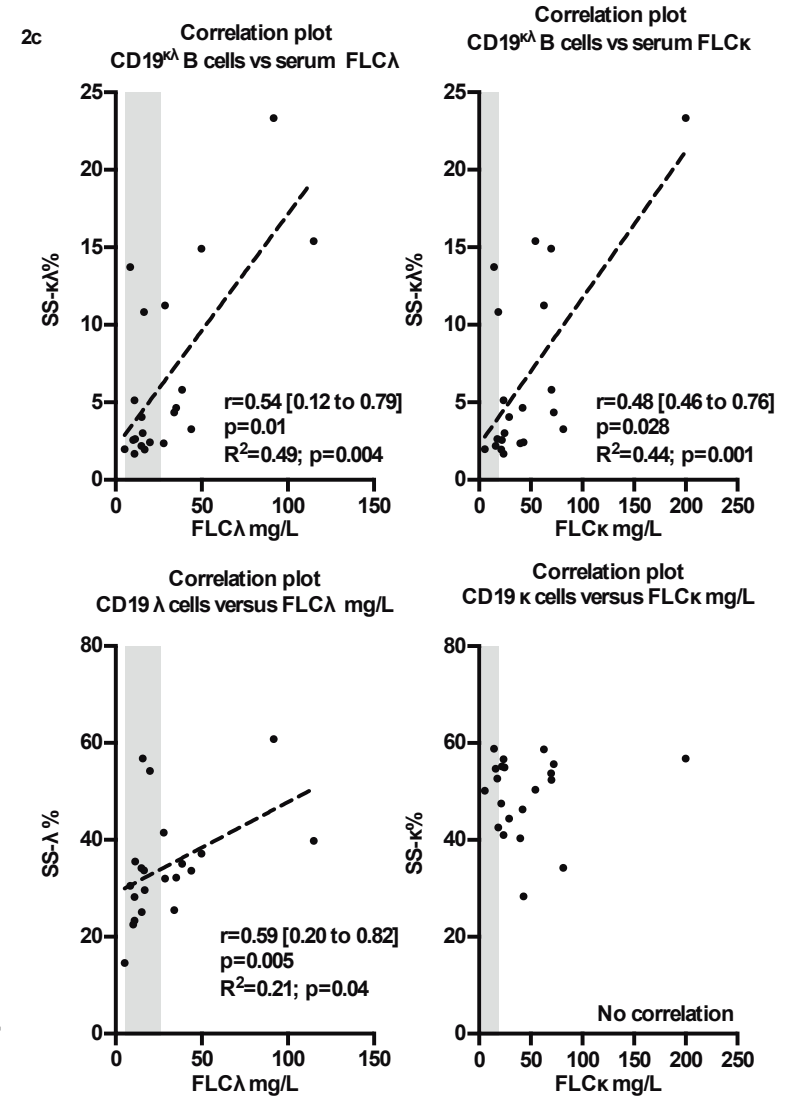
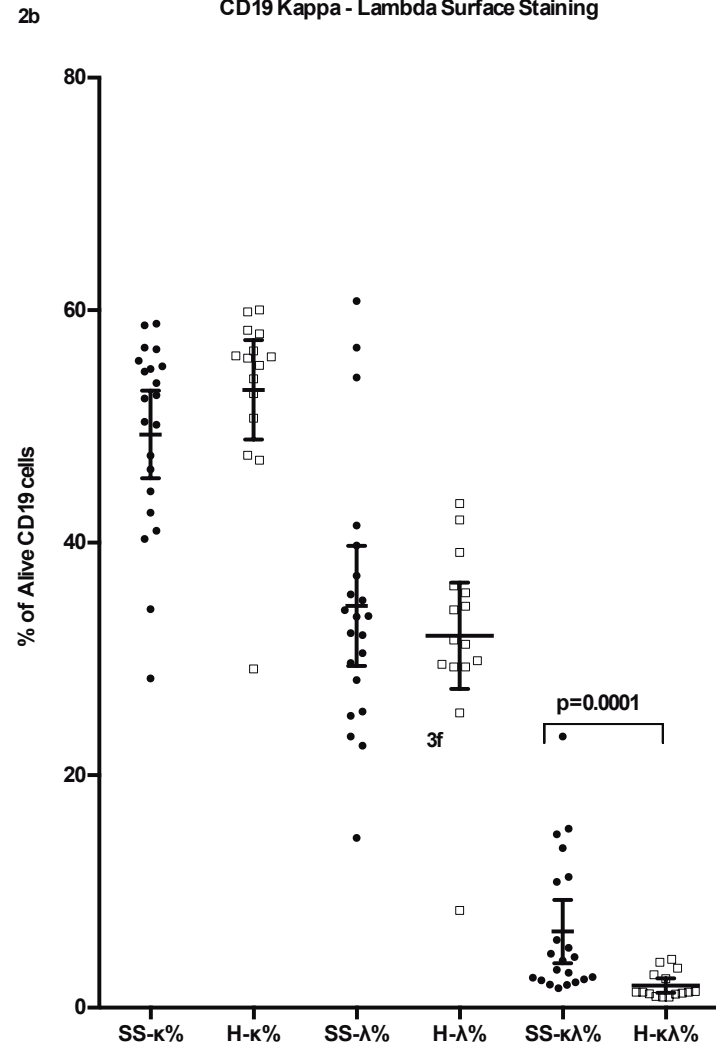
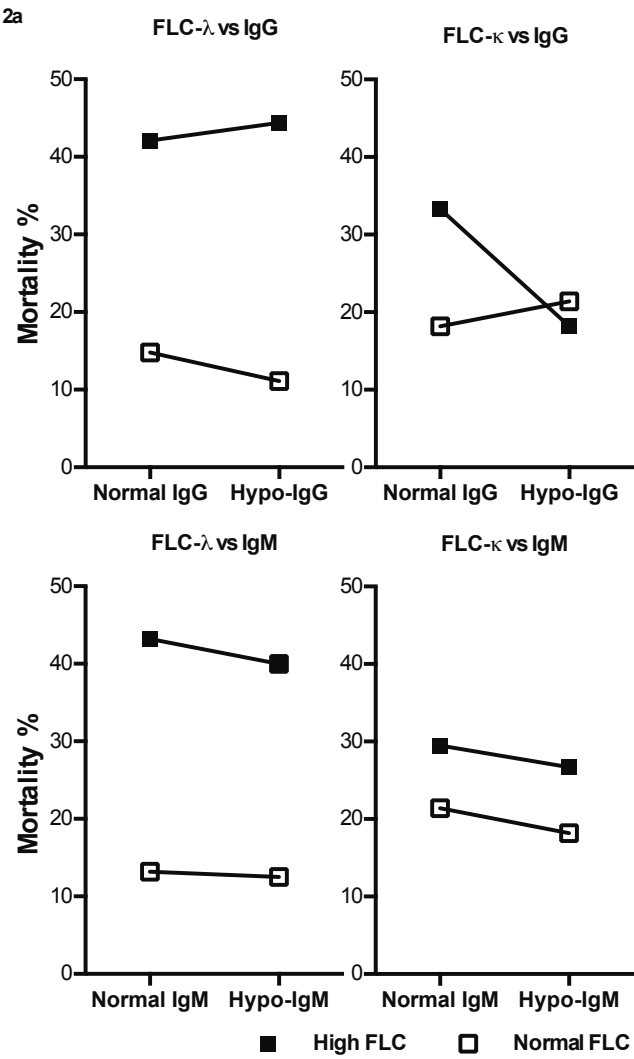


Table-1: Hypothetical relationships between measured concentrations of immunoglobulins and free light chains and enrichment for trial design

Abnormality	Normal immunoglobulin	Low Immunoglobulin
Normal FLC	<p>Scenario A</p> <p>Production and consumption of immunoglobulin light and heavy chains are in balanced equilibrium</p>	<p>Scenario B</p> <p>Immunoglobulin consumption and/or loss exceeds assembly</p>
Raised FLC	<p>Scenario C</p> <p>Effective immunoglobulin assembly meets consumption and loss demands, but there is excess light chain production</p>	<p>Scenario D</p> <p>Rapid immunoglobulin consumption and/or excessive loss with ineffective immunoglobulin assembly results in a large excess of light chain production</p>

Table-2: Admission day cohort characteristics

Variable	Survivors (N=74)	Non-survivors (N=27)
¹ Age years	62.3 (12.6)	71.9 (14.1)
² Female n (%)	26 (35.1%)	12 (44.4%)
¹ APACHE II Score	18.4 (4.9)	24.0 (5.6)
² Infection site n (%)		
Respiratory	46 (62.2%)	19 (70.4%)
Intra-abdominal	12 (16.2%)	3 (11.1%)
Wound and Soft tissue	7 (9.5%)	3 (11.1%)
Urosepsis	6 (8.1%)	1 (3.7%)
Meningitis	1	-
Osteomyelitis	1	-
Unknown	1	1
³ WCC count	14.6 (9.9, 19.0)	13.6 (10.3, 21.8)
³ CRP	148 (74, 278)	131 (93, 248)
³ Total SOFA score	7 (5, 9)	8 (6, 11)
³ Immunoglobulin concentrations		
IgG g/l	7.2 (4.7 – 10.2)	8.0 (5.4 – 10.5)
IgA g/l	2.3 (1.5 – 3.0)	2.2 (1.6 – 3.2)
IgM g/l	0.6 (0.4 – 0.8)	0.7 (0.4 – 1.2)
³ Free light chain concentrations		
FLC-Lambda mg/l	23.2 (15.8 – 36.3)	29.6 (22.5 – 43.1)
FLC-Kappa mg/l	33.2 (18.6 – 54.5)	38.8 (26.8 – 55.5)
FLC Kappa/Lambda Ratio	1.3 (1.0 – 1.8)	1.2 (1.0 – 1.5)
³ ICU length of stay	7 (3,12)	12 (6,18)
³ Hospital length of stay	23 (14, 41)	13 (7, 18)

Table-3: Prevalence of different scenarios by survival status in the overall cohort

Parameter		Normal IgG (N=65) Survivors vs non-survivors Reported as %	Low IgG (N=36) Survivors vs non-survivors Reported as %	Normal IgM (N=75) Survivors vs non-survivors Reported as %	Low IgM (N=26) Survivors vs non-survivors Reported as %
FLCλ category	Normal FLC λ (N=54)	31.1% vs 14.8%	32.4% vs 11.1%	44.6% vs 18.5%	18.9% vs 7.4%
	High-FLC λ (N=47)	29.7% vs 59.3%	6.8% vs 14.8%	28.4% vs 59.3%	8.1% vs 14.8%
FLCκ category	Normal FLC κ (N=25)	12.2% vs 7.4%	14.9% vs 11.1%	14.8% vs 11.1%	11.1% vs 12.2%
	High-FLC κ (N=76)	48.7% vs 66.7%	24.3% vs 14.8%	58.1% vs 66.7%	14.8% vs 14.9%

Electronic supplementary material

Shankar-Hari M et al. Increased circulating free light chains in sepsis is associated with immunoglobulin concentrations, higher risk of death and light chain allelic inclusion in circulating B cells

eFigure-1: Pairwise correlation matrix

Figure shows pairwise correlation matrix for FLC κ , FLC λ , FLC γ , IgG, IgA, IgM, inflammation markers (C reactive protein (CRP), WCC (White cell count), creatinine and severity of illness using (APACHE II score (APII) and total sepsis related organ dysfunction assessment score (tSOFA). This analysis reports the Pearson's product-moment correlation between the variables after accounting for multiple testing using Bonferroni's correction. The p-values are presented in grey boxes above and the correlation coefficients are presented in the blue boxes below the blacked out diagonal cells. P values < 0.05 were considered statistically significant. Only significant p-values are reported for clarity, and the corresponding correlation coefficient are highlighted in bold.

eFigure-2: Logistic regression model assessing the relationship between FLC and acute hospital mortality.

To test the interrelationship between admission day FLC and immunoglobulins to acute hospital mortality, we used two logistic regression models with acute hospital mortality as the outcome: **model-1** had high-FLC λ , total APACHE II score, hypo-IgG, and hypo-IgM as binary covariates and **model-2** had high-FLC κ , total APACHE II score, hypo-IgG, and hypo-IgM as covariates.

e-Figure3: Gating strategy to identify B cells with kappa and lambda surface expression.

Following identification of lymphocytes, a single cell gate was applied. This was followed by CD19 (PerCP Cy5.5) vs. live-dead gate (amcyan). The alive CD19 population was then re-gated using lambda (pacific-blue on x axis) and kappa (APC Cy7 on y-axis). To check whether non-specific binding of kappa and lambda fluorochromes were responsible for the dual kappa-lambda positive cells, the CD19 negative alive population was re-gated using lambda (pacific-blue on x axis) and kappa (APC Cy7 on y-axis). The cut offs for the kappa and lambda gates were ascertained by isotype controls as shown.

eTable-1: Admission day characteristics of patients with sepsis where allelic inclusion in B cells were tested

Abbreviations: M-male; F-female; WCC – Total White Cell Count; CRP – C – reactive protein; APACHE II score -Acute physiology and chronic health evaluation score; t-SOFA score – Total Sequential Organ Failure Assessment Score; ICU = Intensive Care Unit; CAP = community acquired pneumonia

eFigure-1: Pairwise correlation matrix

	FLCL	FLCK	FLCR	IgG	IgA	IgM	CRP	WCC	Creatinine	APII	tSOFA
FLCL		<0.0001	NS	<0.0001	0.03	NS	NS	NS	<0.0001	NS	NS
FLCK	0.727		0.0009	<0.0001	NS	NS	NS	NS	<0.0001	NS	NS
FLCR	-0.183	0.414		NS	NS	NS	NS	NS	NS	NS	NS
IgG	0.476	0.496	-0.017		NS	0.04	NS	NS	NS	NS	NS
IgA	0.335	0.185	-0.200	0.300		NS	NS	NS	NS	NS	NS
IgM	0.076	0.076	-0.013	0.332	0.023		NS	NS	NS	NS	NS
CRP	-0.036	-0.091	-0.052	-0.077	-0.118	-0.028		NS	NS	NS	NS
WCC	-0.063	0.013	0.078	0.242	0.104	0.244	0.185		NS	NS	NS
Creatinine	0.469	0.581	0.255	0.130	0.042	-0.091	-0.023	-0.043		NS	0.01
APII	0.137	0.143	0.061	0.094	0.195	-0.138	-0.033	0.184	0.212		<0.0001
tSOFA	0.191	0.319	0.315	0.030	-0.053	-0.028	-0.180	-0.092	0.366	0.479	

eFigure-2: Logistic regression model assessing the relationship between FLC and acute hospital mortality

. *** Model-1 – abnormal FLCL category

. logistic hosp_m APACHEII_Score IgGCAT IgMCAT FLCLCAT

Logistic regression

Log likelihood = -44.642713

Number of obs = 101

LR chi2(4) = 27.99

Prob > chi2 = 0.0000

Pseudo R2 = 0.2387

hosp_m	Odds Ratio	Std. Err.	z	P> z	[95% Conf. Interval]	
APACHEII_Score	1.215561	.0653236	3.63	0.000	1.094042	1.350579
IgGCAT	.9231006	.5848797	-0.13	0.900	.2666397	3.195753
IgMCAT	.6464151	.4260445	-0.66	0.508	.1776212	2.352493
FLCLCAT	3.985089	2.308288	2.39	0.017	1.280547	12.40168
_cons	.0030006	.0038824	-4.49	0.000	.0002376	.0378922

. *** Model-2 Abnormal FLCK category

. logistic hosp_m APACHEII_Score IgGCAT IgMCAT FLCKCAT

Logistic regression

Log likelihood = -47.424497

Number of obs = 101

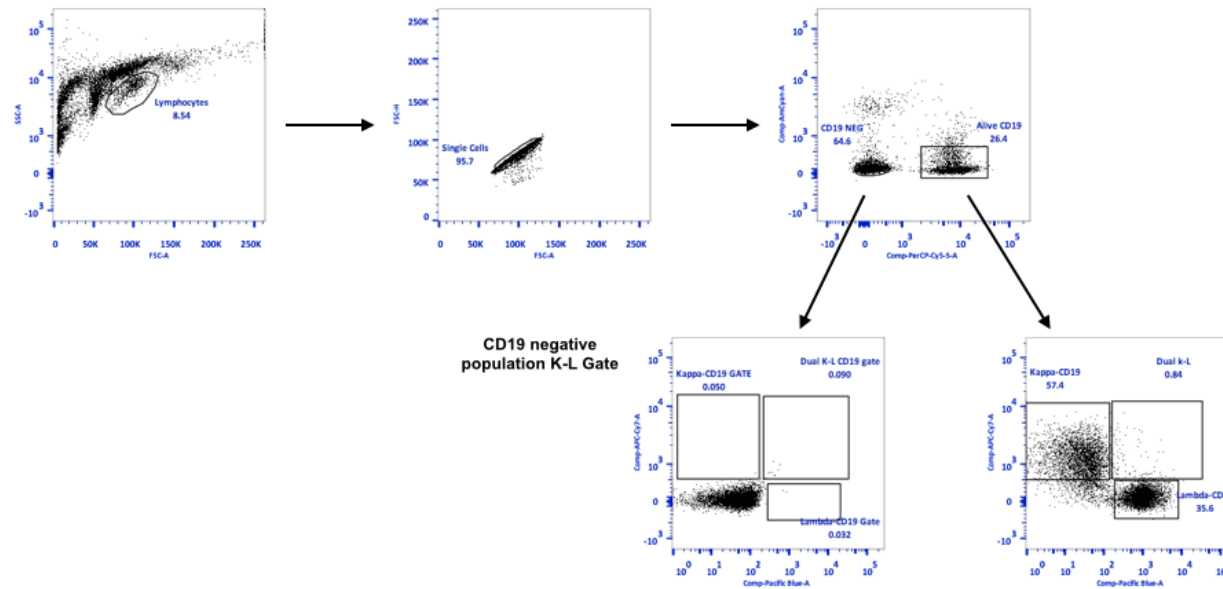
LR chi2(4) = 22.43

Prob > chi2 = 0.0002

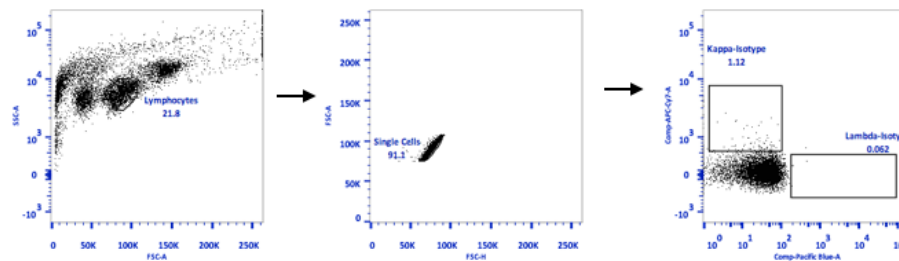
Pseudo R2 = 0.1912

hosp_m	Odds Ratio	Std. Err.	z	P> z	[95% Conf. Interval]	
APACHEII_Score	1.226188	.0632714	3.95	0.000	1.108243	1.356685
IgGCAT	.6751069	.409152	-0.65	0.517	.2058247	2.214357
IgMCAT	.7062794	.4534791	-0.54	0.588	.2006539	2.486025
FLCKCAT	1.707432	1.164346	0.78	0.433	.4486266	6.498331
_cons	.0039778	.0054098	-4.06	0.000	.0002767	.0571853

eFigure-3: Gating strategy to identify B cells with kappa and lambda surface expression



Isotope control gates for cut offs



eTable-1: Admission day characteristics of patients with sepsis where allelic inclusion in B cells were tested

Case	Age year	Sex	Infection	WCC 10 ³ /μL	CRP mg/L	APACHE II score	t-SOFA score	Outcome
CSII007	68	M	CAP	26.3	176	21	8	Alive
CSII008	71	M	CAP	11.4	205	16	7	Alive
CSII010	64	M	Cellulitis of foot and CAP	22	100	18	6	Alive
CSII011	89	F	CAP	17.4	87	15	7	Died
CSII012	61	F	CAP	27	25	19	10	Alive
CSII013	51	F	CAP	18.4	320	24	11	Alive
CSII014	66	M	CAP	1.9	90	17	10	Alive
CSII020	44	M	CAP	34.6	300	14	7	Alive
CSII021	64	M	CAP	11.6	32	27	7	Alive
CSII023	74	M	CAP	4	28	18	12	Alive
CSII024	73	F	Intra- abdominal sepsis	14.6	317	19	6	Alive
CSII026	40	M	CAP	22.6	234	18	3	Alive
CSII031	47	F	CAP	20.9	8	16	3	Alive
CSII032	81	M	CAP	8.6	161	26	9	Alive
CSII033	18	M	CAP	24.9	310	21	13	Alive
CSII035	71	F	Aspiration pneumonia	23.9	170	27	9	Alive
CSII037	43	F	Urosepsis	12.5	511	13	5	Alive